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Investigations of spectrin–lipid interactions using fluoresceinphosphatidylethanolamine as a membrane probe

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Abstract

The binding of human erythrocyte spectrin to large unilamellar vesicles (LUVET) formed by the extrusion technique has been studied using fluoresceinphosphatidylethanolamine (FPE) as a reporter of electrostatic membrane potential. Spectrin aliquots were added to a suspension of FPE-labelled LUVETs to elucidate both the type of charge involved and the dissociation constants for spectrin binding to various lipids. All binding experiments showed serial increases in FPE fluorescence intensity upon serial additions of spectrin, indicative of increasing positive charge at the membrane surface. This proves for the first time that although exhibiting an overall net negative charge, spectrin binds to lipid surfaces by presenting positive charges to the lipid surface. Binding curves were obtained from the change in fluorescence intensity upon each spectrin addition and analysed to determine dissociation constants. A K_d of 0.14 ± 0.12 μM was found for spectrin binding to FPE-labelled phosphatidylcholine/phosphatidylserine (PC/PS) LUVETs at 22°C in high salt conditions. A similar K_d of 0.17 ± 0.11 μM was obtained for spectrin binding to neutral LUVETs composed of PC. However, binding was found to be much weaker for PC/PS LUVETs under low salt conditions with a K_d of 1.22 ± 0.48 μM.

Keywords: Erythrocyte; Cytoskeleton; Lipid analogue; Membrane; Fluorimetry

1. Introduction

Spectrin is the predominant component of the erythrocyte cytoskeleton that adds strength to the cell membrane and helps maintain the erythrocyte shape. Erythroid spectrin is a dimer consisting of α and β subunits with molecular weights of 270 [1] and 246 kDa [2], respectively. Spectrin dimers can form higher oligomers by head-to-head interactions, with tetramers being predominant within intact erythrocytes [3]. The α- and β-subunits of spectrin exhibit homology, both sharing a common 106 amino acid repeat motif that forms a structurally identical barrel, composed of 3 α-helices [4–6]. The subunits interact along their length to form a coiled-coil like rod structure of ~100 nm [6–9] that can compress and expand together.

It is well documented that the erythrocyte cytoskeleton is bound to the erythrocyte membrane through interactions between both cytoskeletal proteins and transmembrane proteins [10,11]. Spectrin is coupled to the transmembrane protein, band 3, via ankyrin and to another transmembrane protein, glycophorin C, via protein 4.1. Spectrin is also linked to

Abbreviations: FPE, fluoresceinphosphatidylethanolamine; LUVET, large unilamellar vesicles formed by extrusion technique; PC, phosphatidylcholine; PS, phosphatidylserine

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vertices composed predominantly of actin oligomers, protein 4.1 and other accessory proteins. Spectrin has also been shown to bind to various lipid surfaces with differing affinities.

Early spectrin–lipid binding studies suggested that spectrin bound to both neutral lipids and, with a greater affinity, to negatively charged lipids [12]. It was also believed that a hydrophobic segment of spectrin inserted into the lipid bilayer beyond the surface of the negatively charged head groups [12,13] although this is no longer thought to be the case under physiological conditions. The first determination of dissociation constants for spectrin–lipid interactions gave K_d values of 0.71 and 2.7 μM for PS/PE and PS/PC, respectively, at pH 6.0 [13].

Since these original studies, many different techniques have been utilised to study spectrin–lipid interactions and these have shown varying results depending on method and lipid type. The majority of studies have shown that spectrin binds to negatively charged lipid surfaces with a K_d between 0.1–0.5 μM [12–19]. However, whilst some reports show that the dissociation constant of spectrin for negatively charged lipids is greater than its affinity for neutral lipids [12–15], others report similar dissociation constants for both negatively charged and neutral lipids [20]. For neutral lipids, a K_d of 0.1 μM was found [18] and a K_d of 0.15 μM using bovine spectrin [19].

The significance of such spectrin–lipid interactions is still unclear. Early studies suggested that spectrin–lipid interactions helped maintain the lipid asymmetry of the erythrocyte membrane but this is no longer thought to be the case since the role of an ATP-driven lipid translocase was elucidated [21–23]. Other possible roles for spectrin–lipid interactions include the further strengthening and shape regulation of the cell membrane or to direct and concentrate unbound spectrin molecules at the inner lipid leaflet to facilitate protein–protein interactions [20].

The use of the fluorescent lipid analogue, fluoresceinphosphatidylethanolamine (FPE), as a real-time probe for studying protein–membrane interactions has recently been described [24,25]. FPE has two major structural components, the phosphatidylethanolamine segment that enables the probe to be readily incorporated into lipid bilayers of liposomes and a fluorescein head group. The xanthene ring system of FPE can undergo protonation or deprotonation, which results in a change in the fluorescence emission. The pK_{app} is dependent on the membrane surface potential which is altered by changes in the nature and concentration of electrolytes or by binding of charged molecules to the membrane surface. Addition of calcium ions to a solution of FPE-labelled vesicles, for example, results in an increase in fluorescence intensity. This is because an increase in positively charged ions reduce the magnitude of the negative electrostatic potential, which in turn results in an increase in the apparent pK of FPE leading to deprotonation of the xanthene ring system and an increase in fluorescence intensity [24].

The properties of FPE appear to make it an ideal tool for the study of spectrin–lipid interactions. The change in fluorescence intensity of FPE-labelled liposomes upon spectrin binding can demonstrate the type of charge interactions involved by directly reporting the nature of the charges being presented to the lipid surface by spectrin molecules. Serial additions of spectrin to FPE labelled liposomes could also enable the dissociation constants of spectrin–lipid interactions for different lipid surfaces to be calculated. The FPE technique has the added advantages that spectrin does not have to be modified by the addition of covalent labels and no separation of spectrin–lipid complexes is required.

With so many differing results concerning spectrin–lipid interactions, further studies are required to elucidate the affinities of spectrin for different lipids. Here we report experiments with FPE that monitors surface potential changes upon spectrin binding and hence provide further information on the nature of spectrin–lipid interactions and a novel method of determining dissociation constants.

2. Materials and methods

2.1. Materials

A23187 was purchased from Fluka, Switzerland. PC and PS were purchased from Lipid Products, UK. FPE was both kindly donated by Professor P. O'Shea and co-workers or purchased from Sigma. Bradford reagent was purchased from Pierce and Warriner, UK. Whole fresh blood was obtained from donors on the day of purification.

2.2. Purification of erythrocyte spectrin

Spectrin was extracted from freshly drawn erythrocytes, purified according to Takeshita et al. [16] and its purity checked by 7.5% SDS–PAGE according to Laemmli [26]. Spectrin was stored in 20% sucrose, 7.5 mM sodium phosphate, 1 mM EDTA, 1 mM sodium azide, 0.1 mM DTT pH 7.5 at 4°C. Spectrin concentrations were measured by Bradford assay [27], using a correction factor of 1.24 BSA equivalents that was established by comparison with absorbance measurements using $\varepsilon_{280}(1\%) = 10.1$.

2.3. Manufacture of LUVET

The method followed that of Wall et al. [24]. Briefly, lipids and FPE, dissolved in chloroform and methanol, were mixed together in desired quantities in a round bottomed flask. The lipid mixture was dried whilst hand swirling under a stream of argon gas. Once dry, the mixture was resuspended in chloroform, dried as before and finally rehydrated in the desired buffer (either 100 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, 1 mM DTT, 1 mM sodium azide pH 7.2 (buffer A) or 5 mM sodium phosphate pH 7.2 (buffer B) to a final lipid concentration of 2.5 mg/ml. The lipid solution was left for 30 min under argon, with intermittent shaking before freeze–thawing five times. The freeze–thawed mixture was passed 10 times through an extruder fitted with two 0.1 μm pore diameter polycarbonate Nucleopore membranes under 25 bar nitrogen gas. The liposomes were then stored at 4°C, under argon and in the dark for no longer than 3 days.

Correct incorporation of FPE was checked by testing the fluorescence change upon the addition of 1 M $CaCl_2$ to a final concentration of 6 μM, followed by the fluorescence change brought about by the addition of calcimycin (A23187 calcium ionophore) [24].

2.4. Spectrin–lipid binding assay

FPE-labelled LUVETs were diluted, typically to around 8.3 μg/ml in the desired buffer and placed in a 3 ml fluorometric cuvette. Fluorescence was recorded using a Shimadzu RF5000 spectrofluorimeter; the cuvette contents were controlled to 20°C and stirred magnetically. The excitation λ was set at 492 nm, emission λ at 516 nm, the excitation slit was set to 5 nm, the emission slit to 10 nm and analyte sensitivity set to high. Fluorescence recordings were taken every second.

Aliquots of spectrin (dialysed in the same buffer as the LUVET suspension and concentrated to ~4 mg/ml just prior to the measurements) were added to the cuvette typically at 1 min intervals whilst being stirred and the change in fluorescence recorded. The total amount of spectrin that could be added was limited as high spectrin concentrations became viscous. Control experiments were undertaken to test for scattering by LUVETs without FPE and for dilution using FPE-labelled LUVETs and addition of spectrin-free buffer.

2.5. Analysis of spectrin–lipid binding curves

The fluorescence intensity was averaged over typically 56 s for 1 min intervals between additions (which also yielded standard deviations seen in Figs. 2–4). The readings were corrected for dilution, using a theoretical dilution factor and for scattering (typically in the order of 0.05%). Fluorescence intensities were then standardised so that the initial fluorescence intensity was equal to zero.

Fluorescence change was plotted against spectrin concentration and fitted by non-linear least-squares analysis to

$$F = ([S]F_{max})/(K_d + [S]) \quad (1)$$

where [S] is the spectrin concentration, F is the fluorescence change and F_{max} the maximum fluorescence change. Each experimental run was fit individually and results averaged to give a final K_d for each experimental condition. The use of Eq. 1 was justified by performing some experiments at different lipid concentrations (4–10 μg/ml) and finding that these yielded similar binding constants.

3. Results and discussion

The interaction of spectrin with lipids was studied with two different lipid mixtures and in two buffer systems. Each addition of spectrin to FPE-labelled vesicles caused an increase in fluorescence intensity; a typical example is shown in Fig. 1. The fluores-

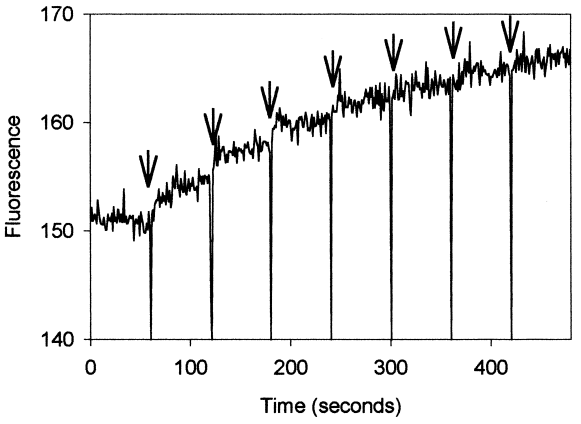

Fig. 1. FPE fluorescence intensity (arbitrary units) observed upon addition of 20 μl aliquots of 7 mg/ml spectrin to FPE-labelled PC/PS LUVETs suspended in low salt (buffer B). Each addition is indicated by an arrow. The interruption in the fluorescence measurements was caused by the closure of the fluorimeter shutter during sample additions. Fluorescence was measured at 516 nm after excitation of the sample at 490 nm.

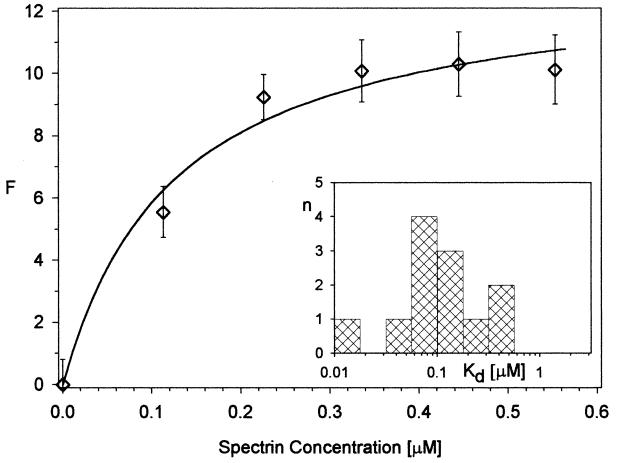

Fig. 2. A typical individual binding curve for spectrin binding to FPE-labelled PC/PS LUVETs suspended in high salt (buffer A). *F* is fluorescence change in arbitrary units. The change in fluorescence has been corrected both for dilution and scattering artefacts and normalised to the initial fluorescence before addition of spectrin. The data were fitted by a non-linear least squares analysis and K_d found to be 0.13 ± 0.04 μM; the values obtained in all measurements are shown inset as a histogram of *n* values on a $\log(K_d)$ axis.

cence intensity changes were fitted by binding curves from which the K_d of spectrin binding under varying conditions was determined (Figs. 2–4 and Table 1). Spectrin was found to bind to negatively charged vesicles (140:60:1 PC:PS:FPE) in high salt (buffer A), as successive additions of spectrin caused the FPE fluorescence to increase. The mean K_d from 12 individual determinations was 0.14 ± 0.12 μM and the distribution of values is shown inset in Fig. 2. This dissociation constant is in good agreement with other published results [14,16–18], for spectrin–lipid binding which have shown spectrin–PS lipid binding with a K_d of between 0.1–0.5 μM.

With neutral vesicles containing only PC and 0.5% FPE in buffer A, spectrin additions caused similar fluorescence changes to those found with the negatively charged vesicles. The mean K_d from seven individual determinations was 0.17 ± 0.11 μM, which was similar to that found for spectrin–PC/PS lipid binding and is in good agreement with other binding studies [18–20]. Bitbol et al. [18] found a K_d of ~ 0.1 μM for spectrin–DMPC and spectrin–DMPC/DMPS binding and Bialkowska et al. [19] found a K_d of 0.1 μM for spectrin (bovine)–PC binding and 0.15 μM for PS/PE (3:2) binding. However, other published reports suggest that spectrin–PC lipid binding is much weaker [12,28] or negligible [14]. Spectrin–PC binding may not have been realised by these workers. Bonnet and Begard studied the fluorescence quenching of a fluorescent probe covalently attached to spectrin. Spin-labelled PC and PS lipids were then used to quench probe fluorescence upon spectrin–lipid binding. Incubation of spectrin with PC vesicles showed no effect, however incubation of spectrin with PS was shown to bind with an affinity of ~ 0.3 μM. The failure to observe spectrin–PC binding may have resulted from interference with the interaction by the fluorescent probe.

Table 1
Average spectrin–lipid dissociation constants

Lipid type	Buffer system	$K_d \pm$ S.D. (μM)	No. of experiments
PC/PS (7:3)	high salt (buffer A)	0.14 ± 0.12	12
PC	high salt (buffer A)	0.17 ± 0.11	7
PC/PS (7:3)	low salt (buffer B)	1.22 ± 0.48	6

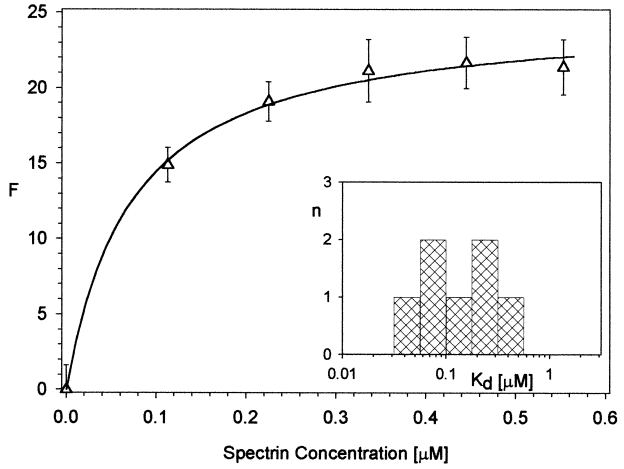

Fig. 3. A typical individual binding curve for spectrin binding to FPE-labelled PC LUVETs suspended in high salt (buffer A). F is fluorescence change in arbitrary units. The change in fluorescence has been corrected both for dilution and scattering artefacts and normalised to the initial fluorescence before addition of spectrin. The data were fitted by a non-linear least squares analysis and the K_d found to be 0.08 ± 0.01 μM; the values obtained in all measurements are shown inset as a histogram of n values on a $\log(K_d)$ axis.

In low salt conditions using buffer B and negatively charged vesicles (140:60:1 PC:PS:FPE), spectrin was also shown to bind, as successive additions also caused a fluorescence increase (Fig. 1) but with a much higher K_d of 1.22 ± 0.48 μM from six individual determinations. This was nearly 10 times higher than that of spectrin binding in high salt which was found to be 0.14 ± 0.12 μM. The increased dissociation of spectrin–lipid interactions in low salt conditions was not unexpected as spectrin can be purified from the erythrocyte by low salt extraction. The finding is also in good agreement with MacDonald [17] where dissociation of spectrin from PS vesicles was shown to be enhanced at low salt concentrations of 3 mM KCl, pH 7.

The increased K_d may be a result of several factors. At low ionic strength, spectrin molecules are thought to be more extended, increasing from ~70 nm in length in high salt to ~200 nm in low salt [7,8,29]. It has been suggested that this conformational change would bring the negatively charged residues closer to the membrane surface which would result in an increase in electrostatic repulsion compared to spectrin in high salt [17]. It is still unclear whether spectrin binds to lipid surfaces via electrostatic, hydrophobic or combined interactions. If interactions are electrostatic, decreasing salt concentration should result in decreasing dissociation, however this may be countered by the increased repulsion created by spectrin conformational changes.

Under high salt conditions, both spectrin–PC and spectrin–PC/PS binding curves were similar and appeared to approach saturation with the changes in fluorescence decreasing when higher spectrin concentrations were aliquoted to the vesicle suspension (Figs. 2 and 3). However low-salt spectrin binding curves suggested that spectrin–lipid binding had not approached saturation (Fig. 4). The increased K_d of spectrin–lipid binding in low salt compared to that in high salt is one possible reason why spectrin–lipid binding did not approach saturation. However, MacDonald [17] showed that increased temperatures resulted in an increased spectrin dissociation from PS-vesicles in high salt coupled with an increase in saturation. It may be that the increased K_d is also associated with a higher degree of saturation in low salt as a result of conformational changes that result in more, lower affinity, spectrin–lipid interactions.

Although binding assays with the same spectrin preparation gave reasonably consistent results, large

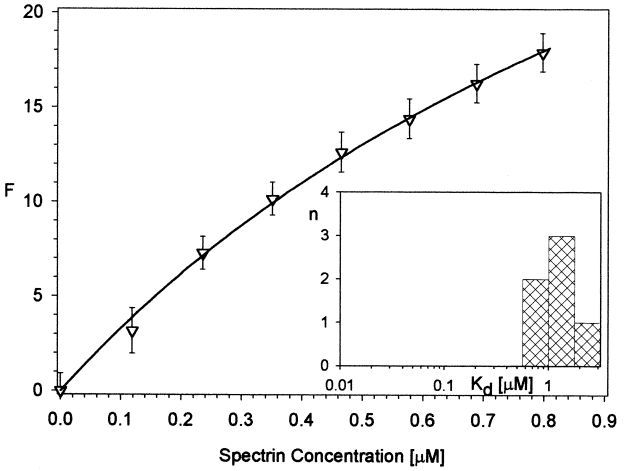

Fig. 4. A typical individual binding curve for spectrin binding to FPE-labelled PC/PS LUVETs suspended in low salt (buffer B). F is fluorescence change in arbitrary units. The change in fluorescence has been corrected both for dilution and scattering artefacts and normalised to the initial fluorescence before addition of spectrin. The data were fitted by a non-linear least squares analysis and the K_d found to be 1.25 ± 0.19 μM; the values obtained in all measurements are shown inset as a histogram of n values on a $\log(K_d)$ axis.

standard deviations were found between different spectrin preparations (see insets in Figs. 2–4). It would seem unlikely that FPE was the cause of the variations, as FPE performance tests described in Section 2 were routinely performed. Purity of spectrin was also routinely tested by SDS–PAGE both before and after binding assays and showed no signs of degradation or impurities. Spectrin however may have undergone structural changes that are not evident, but alter the spectrin molecule's interactions with lipids. There is no functional assay of spectrin and so although not degraded, structural changes could have occurred during purification. Spectrin can also undergo phosphorylation [30]; the phosphorylation state of spectrin was not tested and may have contributed to the experimental variations.

Possibly the most significant finding of these spectrin–lipid interaction studies was that spectrin binding to FPE-labelled vesicles, regardless of lipid type or ionic strength gave rise to a net increase in fluorescence intensity. The properties of FPE are such that, when the pH of the bulk solution remains constant, an increase in fluorescence intensity is indicative of an increased number of positively charged residues at the membrane surface.

Spectrin has a net negative charge at pH > 5.6 [31] and it has been speculated that spectrin has positive binding pockets that enable spectrin–PS interactions [15]. This is the first time that spectrin has been conclusively shown to bring positive charges to lipid surfaces. However, these positive charges may not be directly involved in lipid binding as spectrin binds to both neutral lipid surfaces and negatively charged lipid surfaces with a similar affinity. If spectrin bound to the lipid surface through direct electrostatic interactions, it would be expected to have a higher affinity for PS than PC, but spectrin has been found here to have a similar affinity for both lipid types. It may be that spectrin binds to the lipid surface via dipolar interactions with both neutral and negatively charged lipid head groups, as postulated by Bitbol et al. [18] or that the positive charges are not involved with direct binding but are brought nearer to the surface upon spectrin–lipid binding.

When spectrin was added at low concentrations, the fluorescence changes were more complicated. The net change in fluorescence intensities reached a steady positive value, however an initial fluorescence decrease was followed by a slower fluorescence increase that reached equilibrium after approximately 40 s (Fig. 5). This could be a result of spectrin binding via hydrophobic interactions that initially bring nearby negatively charged areas along the spectrin length into close proximity of the lipid surface. It would appear that spectrin then reorganises so as to bring positive charges closer to the lipid surface. Alternatively, spectrin may rearrange so as to move the negative charges away from the lipid surface. It is unclear why this effect is only observed at low spectrin concentrations.

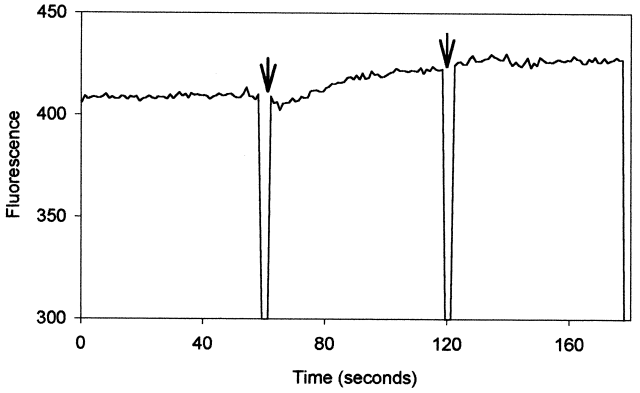

Fig. 5. FPE fluorescence intensity (arbitrary units) observed upon addition of two 20 μl aliquots of 0.5 mg/ml spectrin to FPE-labelled PC/PS LUVETs suspended in high salt (buffer A) and corrected for both dilution and scattering. The addition of each aliquot is indicated by an arrow. Fluorescence was measured at 516 nm after excitation of the sample at 490 nm.

Spectrin–lipid interactions in high salt have a low dissociation constant which although agreeing with previous publications, seems counter-intuitive when considering that only weak spectrin–lipid interactions have been postulated. Bitbol et al. [18] suggested that spectrin interacted with PS molecules by a dipolar interaction of spectrin with the lipid–water interface and we ourselves [32] have found that spectrin diffuses very rapidly on the lipid surface which can only be accounted for by very weak spectrin–lipid interactions. However, as has been suggested before [17,32] these results can be explained by the existence of multiple weak lipid interactions along the length of spectrin which would require the simultaneous dissociation of all its binding sites for spectrin to dissociate from the lipid surface resulting in an apparently low dissociation constant. Such interactions would enable spectrin to be directed to the inner lipid

leaflet where it could then be localised and allowed to diffuse so as to undergo further protein–protein interactions with other cytoskeletal proteins.

In conclusion, the present experiments demonstrate that FPE is an effective reporter for spectrin–lipid interactions. The FPE technique should prove useful in future studies; for example stopped flow experiments could be performed to characterise the initial binding events [33]. Both spectrin subunit-lipid and spectrin fragment–lipid interactions could also be studied to further characterise how spectrin interacts with lipids along the length of the spectrin molecule.

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